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Metformin attenuate fibrosis in both acute and chronic doxorubicin cardiotoxicity in rabbits

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Abstract

Doxorubicin is one of the highly effective anti-neoplastic drugs of the anthracyclines family used to treat many pediatric and adult cancers, However the clinical used of doxorubicin is limited due to severe cardiotoxicity side effect. Metformin reducing basal and postprandial glucose levels. The Metformin antihyperglycemic effect seems to reduce cardiovascular death in patients with T2DM, in addition Metformin suggested to have a direct cardioprotective effect which independent on glucose reduction activity. This research aimed to study the protective effect of metformin against doxorubicin cardiotoxicity and fibrosis. **Method**: Thirty six albino rabbits divided to six groups, six rabbits each. Control group, received 2 ml single dose of saline intraperitoneally. Metformin group, received (300 mg/kg/day, every day for 14 days) oral solution by gavage. Chronic doxorubicin group, received (4mg/kg, twice a week, cumulative dose: 16 mg/ kg) intraperitoneally. Acute doxorubicin group received (16 mg/ kg single dose) intraperitoneally. Chronic doxorubicin+ metformin group received doxorubicin(4 mg/kg, twice a week intraperitoneally and metformin(300 mg/kg/day, for 14 days, starting three days prior to doxorubicin) orally. Acute doxorubicin+ metformin group received doxorubicin(16 mg/kg single dose) intraperitoneally and metformin (300 mg/kg/day, for 14 days, starting three days prior to doxorubicin) orally. Assessment of serum troponin I, SMAD3 and .RTPCR for TGF-β1 in addition to trichrome stain.

Result: our result showed that pretreatment with metformin significantly ($p<0.05$) decreased the level of serum troponin I, SMAD3 and TGF-β1 in both MET +acute DOX and MET +chronic DOX group in compare with the acute DOX and chronic DOX group, in addition to significantly decreased collagen fiber production.

Conclusion: this study demonstrated that metformin have a protective effect against doxorubicin cardiotoxicity in both acute and chronic induction, by decreasing serum troponin I, SMAD3 /TGF-β1 signalling pathway thereby significantly decreasing the collagen fiber production and fibrosis formation.

Keywords: Metformin attenuate; acute doxorubicin; chronic doxorubicin; cardiotoxicity.

INTRODUCTION

Anthracyclines including daunorubicin, idarubicin, doxorubicin (DOX), and epirubicin, are regarded as the most effective anticancer drugs against acute myeloblastic and lymphoblastic leukemia. DOX in particular have a highly broad spectrum of action including both solid tumors, such as, sarcomas, breast cancer, and also solid tumors in children (e.g. Wilms tumor) and hematological malignancies, such as, Hodgkin disease, leukemia, and non-Hodgkin lymphomas. . Unfortunately, cardiotoxicity is the most toxic consequence of AC[(1)]. Cardiotoxicity-induced by chemotherapy is a dangerous complication that limits the use of chemotherapeutic agents, especially the anthracyclines (AC), since it may lead to the development of the life threatening cardiomyopathy[(2)]. The cardiotoxicity risk increases in patients with hypertension, liver disease, diabetes mellitus, and previous heart diseases [(4)]. The risk also depends largely on the route of administration, duration of chemotherapy (cumulative dose) and the dosage regimen. For example, a concomitance treatment of anthracyclines with other cardiotoxic drugs , such as trastuzumab or paclitaxel, would largely increase the risk of cardiotoxicity that potentially lead to dangerous congestive heart failure[(3)]. Anthracyclines-induced cardiotoxicity is divided into three forms: immediate pericarditis–myocarditis syndrome, an early-onset progressive and the late-onset chronic progressive form. The last two forms are common [(4)]. DOX is a secondary metabolite of Streptomyces peucetius. It is greatly effective anticancer drugs that used to treat many pediatric and adult cancers, e.g. solid tumors, lymphomas, leukemia and breast cancer. DOX have severe toxicities, e.g. hematopoietic suppression, alopecia, extravasation ,nausea and vomiting, yet the cardiotoxicity being the most important. The beginning of this cardiotoxicity may be delayed up to 10 –15 years after stopping of therapy. This cardiotoxicity characterized by a wide range of symptoms extending from asymptomatic changes in ECG to the pericarditis and then to cardiomyopathy[(5)]. The DOX cardiotoxicity differs according to gender, The female have more severe toxicity with more depressed contractility compared with male. The variance in time of onset proposes that several mechanisms can possibly be associated with DOX cardiotoxicity[(6)]. Several mechanisms are indicated in DOX induced cardiotoxicity. DOX-induced cardiomyopathy is largely associated with increasing of oxidative stress, as shown in reactive oxygen species (ROS) induced destruction, e.g. lipid peroxidation, in addition to decreased antioxidants levels and sulfhydryl groups. Another important mechanisms are apoptosis, extracellular matrix remodeling, deterioration of myofibrillar and intracellular dysregulation of calcium level are also related in doxorubicin-induced cardiotoxicity. Furthermore, the cardiotoxicity associated with doxorubicin administration also happened due to changes in endothelin-1 levels, the high energy phosphate pool and disorders in cardiac adrenergic signaling[(7)]. Metformin (Met) is a biguanide (1,1-dimethylbiguanide) commonly used therapy in type 2 diabetes mellitus (T2DM) patients. Met has a good treatment efficacy and safety profile, low cost, and consider as the first-line oral treatment in T2DM in conjunction with lifestyle modification [(8)]. Met has a beneficial effects in both glucose and lipid metabolism. Met acts by decreasing insulin resistance and reducing levels of serum insulin, leading to a reduction of blood glucose level without producing hypoglycemia. So Met act as antihyperglycemic agent and also insulin sensitizer. The Met antihyperglycemic effect seems to reduce cardiovascular death in patients with T2DM [(9)], in addition Met suggested to have a direct cardioprotective effect which independent on glucose reduction activity[(10)]. Metformin has been reported to have cardioprotective effect in addition to reducing basal and postprandial glucose levels, weight loss, and reducing lipid serum levels. Animal model studies of isolated myocardial infarction and HF have shown that Met increases the tolerance of myocardial cells to ischemia-reperfusion injury, and reduce the development of HF after infarction [(11)].

Met decreases the production of ROS in cultured endothelial cells [(12)], and in animal model studies of heart failure[(13)], and also have myocardial protection from oxidative stress induced by H2O2 or TNFα [(14)]. These effect occur by its activation of 5′-adenosine monophosphate-activated protein kinase (AMPK). In addition AMPK have an important role in maintaining cellular or whole body energy homeostasis, So the reduced of ATP during ischemia was significantly decreased by

Met. AMPK also demonstrated to play a significant roles in the genotoxic stress response and in apoptosis regulation [15].

Asensio-Lopez *et al*. confirmed that Met has cardioprotective effect from DOX induced injury and the cardiac adiponectin (APN) system shows an important role[16]. Met increases the circulating levels of APN[17], and also the expression of adiponectin receptors (adipoR1 and adipoR2) [18]. APN is an adipokine synthesized in the adipose tissue, which exerts beneficial effects on the vessel and myocardium cells by binding to its specific receptors and the AMPK activation [19].

The APN and its receptors have been described to be expressed in the myocardium [20], together with its capability to decrease oxidative stress and to support cell survival [19] and even to attenuate the DOX- induced apoptosis [21]. The APN system is involved in various physiological processes such as energy metabolism, vascular physiology and inflammation , by acting directly on the liver, vascular endothelium and skeletal muscle[22]. As well as, APN acts on myocardial cells preventing their death, reducing ischemic injury and helping revascularization.

Cardiac troponin I (cTnI) are regulatory proteins released to the circulation after myocyte damage occurs [23]. The first biomarkers is troponin that known to identify cardiac injury. Troponins are medium sized regulatory proteins act to regulate the contractile elements, myosin and actin . While it is usually undetectable protein, troponins can increase after 2 - 3 hours when the cardiac injury occurs[24]. TnI seems to have better predictive value than TnT with greater sensitivity for detecting cardiac damage induced by AC cardiotoxicity, mainly in the leukemia population [25].

The transforming growth factor β (TGF-β) is the most multifunctional cytokine known. TGF-β exert strong and various effects on several different cell functional types and are implicated in a wide variety of biological cell developmental processes for example embryonic development, proliferation, cell survival, apoptosis, cell growth and differentiation, cellular homeostasis, fibrosis and regulation of inflammatory and immune response [26].

Small mother against decapentaplegic (Smad) are intracellular signaling effectors which are essential for the mediation of the TGF-β intracellular signaling[27]. Today, Smads appear to be involved in a wide range of heart disease , such as HF in the context of cardiac fibrosis and hypertrophy, myocardial infarction, atherogenesis and others. The Smads are implicated in cell growth, morphogenesis, apoptosis, and immune response[28]. In experimental autoimmune myocarditis the major source of cardiac fibrosis triggered by TGF-β may be the heart-infiltrating prominin-1(+) progenitors. TGF-β mediated Smad phosphorylation may modulate heart-infiltrating prominin-1(+) then cell differentiation into fibroblasts [29]. Myocardial fibrosis was associated with up regulations of TGF-β and also the Smad proteins, in addition to myocyte apoptosis [30]. From this, it appears that harmful effects of TGFβ /Smad signaling can obviously be created by development of cardiac fibrosis. Several studies demonstrated an up-regulated expression of TGF-β, Smad2, Smad3, Smad4 and down-regulated expression of Smad7 gene in DOX treatment rat , So TGF-β gene down-regulation leading to suppression of myocardial apoptosis and fibrosis [31].
This study aimed to evaluate the cardioprotective effect of metformin against doxorubicin-induced acute and chronic cardiotoxicity.

METHODS

Animals

Thirty six rabbits were used in this study purchased from animal house - college of veterinary medicine – Alqassim green university. The rabbits were kept in large cages with free access to food and water. The cages were placed in a quiet and temperature controlled room in which a 12:12-hour light-dark cycle was maintained. The weight of the rabbits varied between 0.6 - 2 kg. The rabbits were allowed a ten days acclimatization period before being used in experiments.

Study design

The rabbits divided randomly into six groups, each consisting of six rabbits. The Control group received single dose of saline injection at a dose of 2 ml via intraperitoneal route. The Met group received Met (300 mg/kg/day, every day for 14 days) with gavage. The chronic DOX group received (4mg/kg, twice a week, cumulative dose: 16 mg/ kg) intraperitoneally (chronic induction). The acute DOX group received (16 mg/ kg single dose) intraperitoneally (acute induction). The Met + chronic DOX group received DOX (4 mg/kg, twice a week, cumulative dose: 16 mg/kg) intraperitoneally and Met (300 mg/kg/day, for 14 days, starting three days prior to DOX treatment) orally with gavage. The Met+ acute DOX group received DOX (16 mg/kg single dose) intraperitoneally and Met (300 mg/kg/day, for 14 days, starting three days prior to DOX treatment) orally with gavage.

Induction of cardiotoxicity

Induction of cardiotoxicity carried out by the administration of DOX intraperitoneally in a dose of 16 mg /kg as a single dose for acute cardiotoxicity induction and in a dose of 4 mg/kg, twice a week for two weeks (cumulative dose: 16 mg/kg) for chronic induction [32].

Sample collection and preparation

At the end of ECG recording, the blood (5 ml) collected from each rabbit by using heart puncture , then the blood sample put in plain tube containing gel ,then centrifugation at 3000x for 15 minute for serum preparation . The serum stored at -80°C for ELISA analysis . The rabbits were sacrificed after taking the blood samples by using di-ethyl ether for anesthesia, then the heart removed immediately and washed by distilled water . A small portion of the heart tissue was kept in a sterile tube of normal saline and preserved at -80 º C, which will be used for molecular tests (DNA extraction and RT- PCR), and the other portion kept in 10% buffered neutral formalin to prepare paraffin embedded blocks for trichrome stain assessment.

Statistical analysis

The statistical analysis was performed by using SPSS version 16.0. All results were expressed as mean ± standard deviation (SD). To compare the results between the groups, we used multiple analyses of variance (One way ANOVA test), followed by a post hoc Tukey test. Statistical significant differences was considered when the $p<0.05$ and highly significant differences was considered when the $p<0.001$ for data.

RESULTS

Effect of metformin on cardiac troponin I

Metformin showed a protective effect against troponin I elevation in both acute and chronic DOX toxicity. The descriptive statistics for cardiac troponin I concentration which is represented as mean ± SD (table 3-1) was significantly elevated in both acute and chronic DOX group (252.12 ± 40.79 , 295.33 ± 144.12 pg/ml respectively) in comparison with the control and MET group (126.32 ± 30.91 , 136.42 ± 32.06 pg/ml respectively ; $P < 0.05$). The cardiac troponin I concentration was significantly decreased in both MET +acute DOX and MET +chronic DOX group in compare with the acute DOX and chronic DOX group (130.37 ± 22.30 , 124.48 ± 49.09 pg/ml respectively; $P < 0.05$). as shown in figure (A and B).

Effect of metformin on Smad 3

Metformin showed a protective effect against Smad 3 elevation in both acute and chronic DOX toxicity. The descriptive statistics for Smad 3 concentration which is represented as mean

± SD (table 3-2) was significantly elevated in both acute and chronic DOX group (2.61 ± 0.15 , 3.43 ± 2.42 ng/ml respectively) in comparison with the control and MET group (1.08 ± 0.16, 0.75 ± 0.36 ng/ml respectively; P < 0.05). The serum SMAD 3 concentration was significantly decreased in both MET +acute DOX and MET +chronic DOX group in compare with the acute DOX and chronic DOX group (1.09 ± 0.07, 0.74 ± 0.27 ng/ml respectively; P < 0.05). as shown in figure (2 A and B).

Effect of metformin on transforming growth factor β1

Metformin showed a protective effect against TGFβ1 elevation in both acute and chronic DOX toxicity. The descriptive statistics for DNA load of TGFβ1 concentration which is represented as mean ± SD (table 3-4) was significantly elevated in both acute and chronic DOX group (1861.66 ±408.72, 7237 ± 661.12 copies/gm respectively) in comparison with the control and MET group (186.66 ±51.25, 1008.33 ±149.72 copies/gm respectively ; P < 0.001). The DNA load of TGFβ1 concentration was significantly decreased in both MET +acute DOX and MET +chronic DOX group in compare with the acute DOX and chronic DOX group (230±91.86, 683.33±116.90 copies/gm respectively ; P < 0.001). as shown in figure (3 A and B).

Effect of metformin on histological change by Trichrome stain

Metformin decreased the collagenous fibers production in DOX cardiotoxicity especially in chronic DOX toxicity. The result for all groups illustrated below:

A -control group: the cardiac tissue in control group was normal without any increase in collagen fibers as shown in figure (4 A).

B -MET group: the cardiac tissue in MET group was normal without any increase in collagen fibers as shown in figure (4 B).

C-Acute DOX group: the cardiac tissue in acute DOX group showed very mild increased in collagen fibers as shown in figure (4 C).

D-Chronic DOX group: the cardiac tissue in chronic DOX group showed large increased in collagen fibers as shown in figure (4D).

E-MET +acute DOX group: the cardiac tissue in MET +acute DOX group was normal without any increase in collagen fibers as shown in figure (4 E).

F-MET +chronic DOX group: the cardiac tissue in MET +chronic DOX group showed mild increased in collagen fibers as shown in figure (4 F).

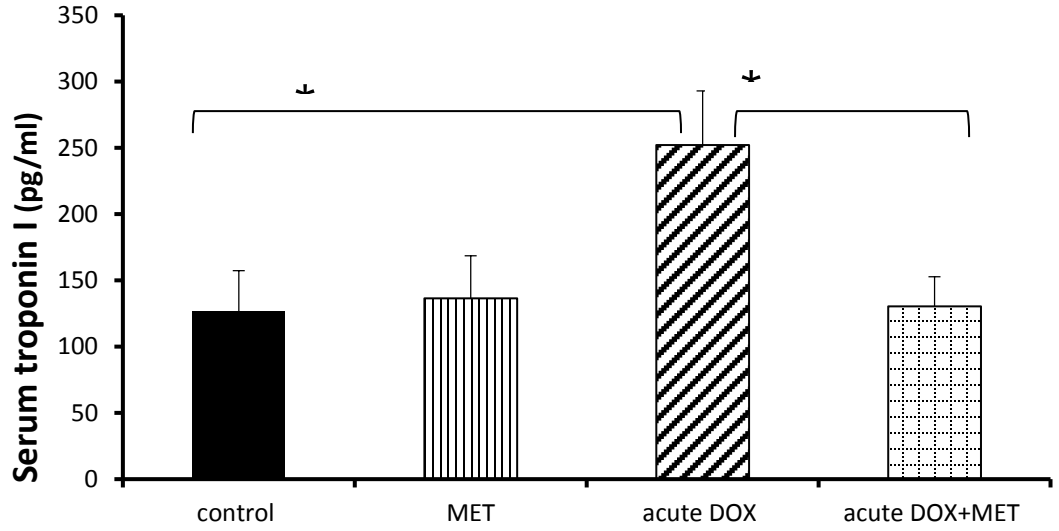

Figure. 1.A Effect of metformin(300 mg/kg) on serum troponin I level in acute DOX cardiotoxicity(16 mg/kg single dose). Each value expressed as mean ±SD. The statistical analysis done by using one way ANOVA followed by Tukey test. * significant difference ($p < 0.05$)

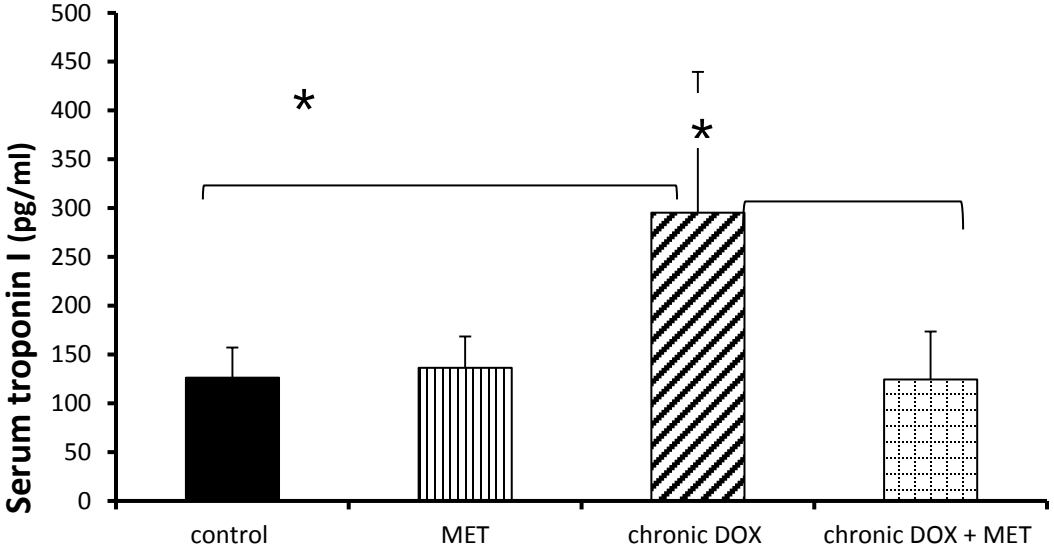

Figure. 1.B Effect of MET (300 mg/kg) on serum troponin I level in chronic DOX cardiotoxicity(4mg/kg twice weekly for two week). Each value expressed as mean ±SD. The statistical analysis done by using one way ANOVA followed by Tukey test. * Significant difference ($p < 0.05$)

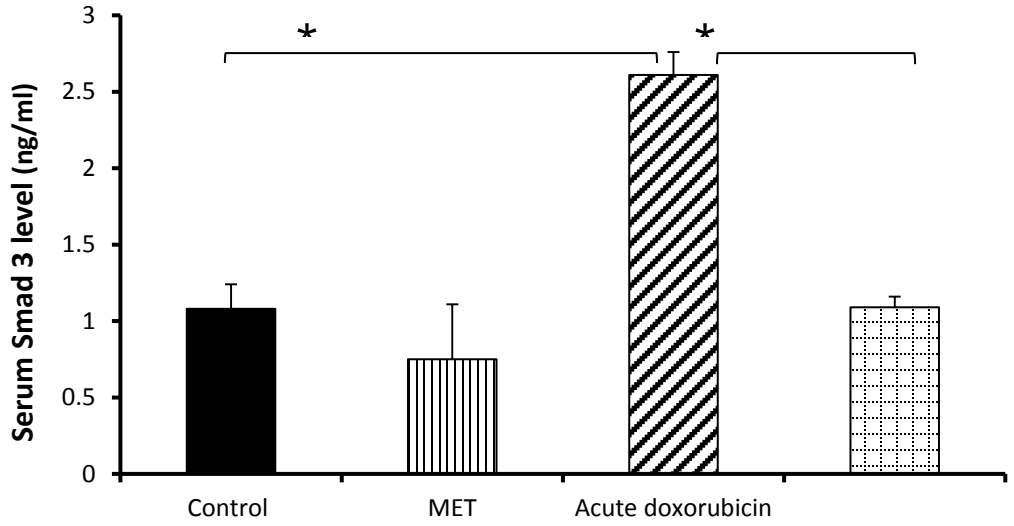

Figure 2.A. Effect of metformin (300 mg/kg) on serum Smad3 level in acute doxorubicin cardiotoxicity (16 mg/kg single dose). Each value expressed as mean ±SD. The statistical analysis done by using one way ANOVA followed by LSD test. *significant difference ($p < 0.05$).

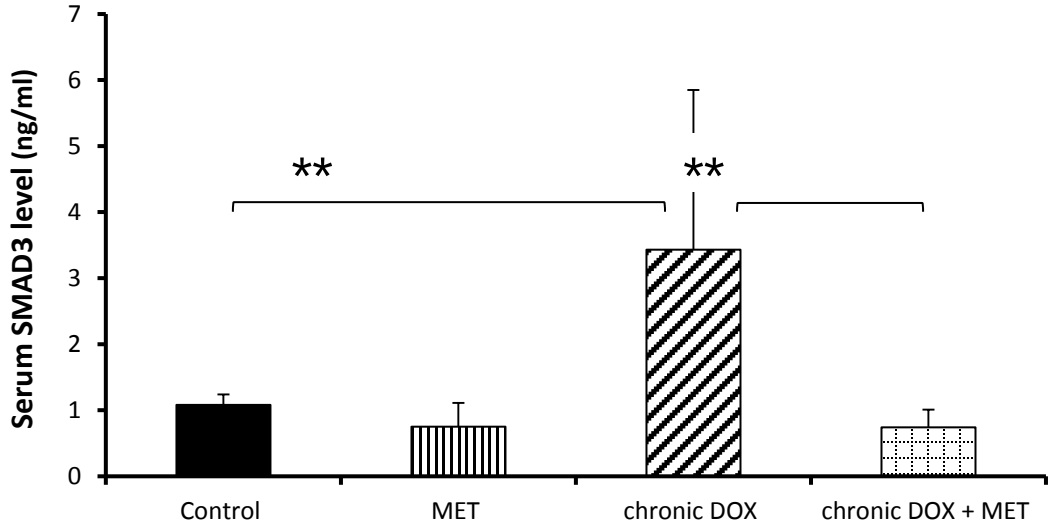

Figure 2.B. Effect of metformin (300 mg/kg) on serum SMAD3 level in chronic DOX cardiotoxicity (4 mg/kg twice weekly for two weeks). Each value expressed as mean ±SD. The statistical analysis done by using one way ANOVA followed by LSD. ** highly significant difference ($p < 0.001$).

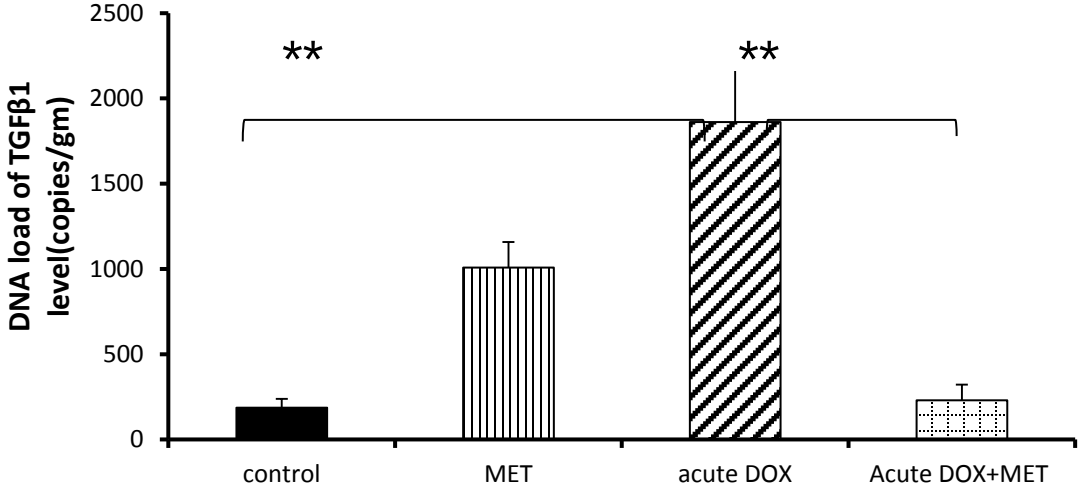

Figure 3.A. Effect of metformin (300mg/kg) on DNA load of TGFβ1 level in acute DOX cardiotoxicity(16mg/kg single dose). Each value expressed as mean ±SD. The statistical analysis done by using one way ANOVA followed by Tukey test. ** highly significant difference ($p < 0.001$)

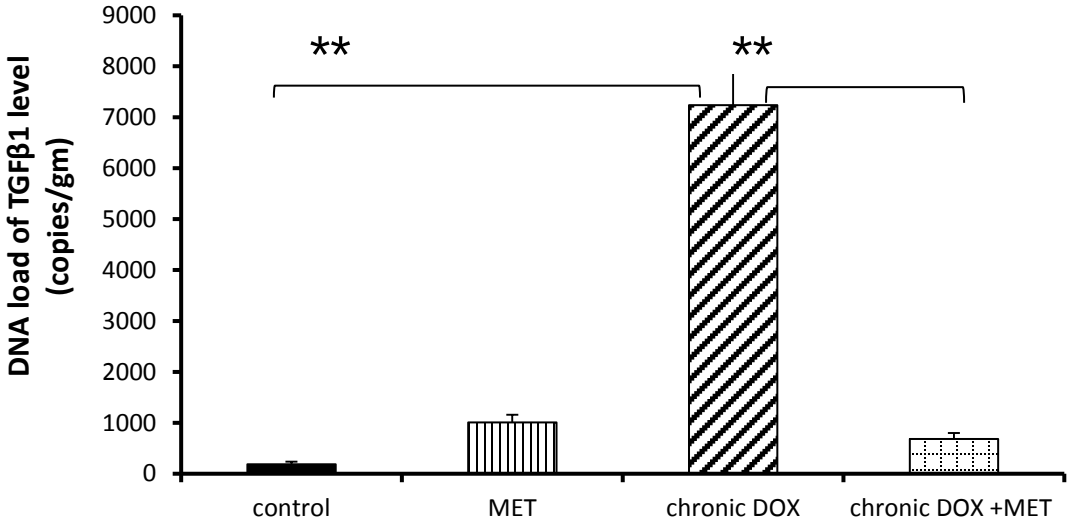

Figure 3. B. Effect of metformin(300mg/kg) on DNA load of TGFβ1 level in chronic DOX cardiotoxicity(4mg/kg twice weekly for two weeks). Each value expressed as mean ±SD. The statistical analysis done by using one way ANOVA followed by Tukey test.
** highly significant difference ($p < 0.001$).

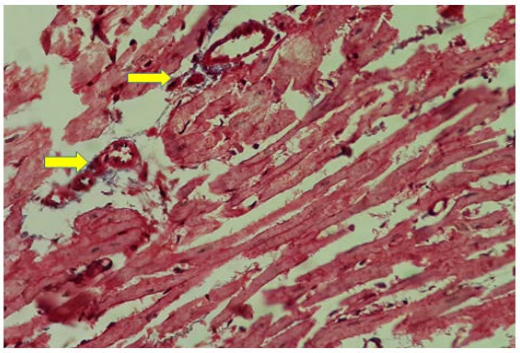

Figure 4.A. Normal saline treated rabbit (control group)showing normal collagenous fibers of the heart, mainly surrounding the small blood vessels (arrows). Trichrome stain, X400.

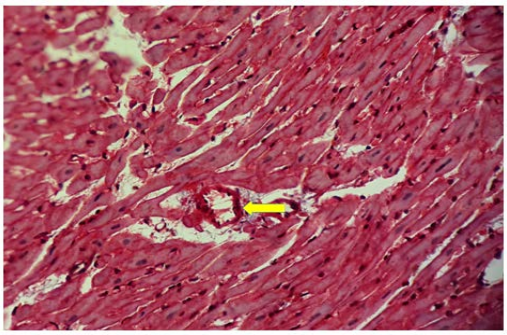

Figure 4.B. Metformin treated rabbit showing normal collagenous fibers of the heart, mainly surrounding the small blood vessels (arrows). Trichrome stain, X400.

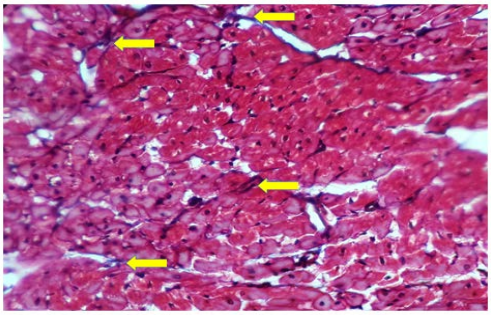

Figure 4.C. acutely DOX treated rabbit, showing very mild increased in collagenous fibers (blue colored collagen fibrous tissue) surrounding myocardial cells. Trichrome stain, X400.

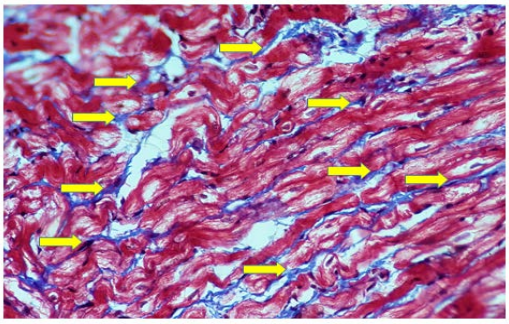

Figure 3.9.D Chronic DOX treated rabbit showing large increase of collagenous fibers (blue colored, collagen fibrous tissue) . Trichrome stain, x 400.

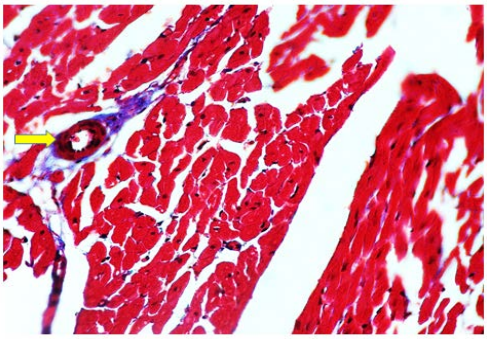

Figure 4.E. MET +acute DOX treated rabbit showing normal collagen fibers of the heart, mainly surrounding the small blood vessels (arrows). Trichrome stain,X400.

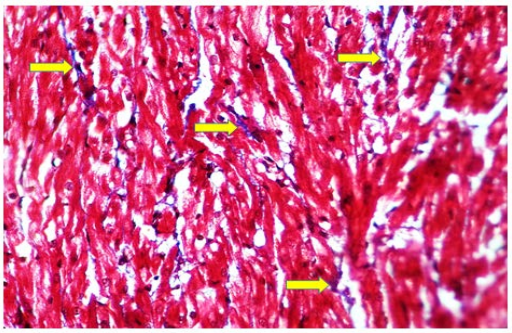

Figure 4.F. MET +chronic DOX treated rabbit showing mild increase of collagen fibers of the heart tissue(blue colored collagen fibrous tissue). Trichrome stain, x 400.

DISCUSSION

Doxorubicin is one of the most anticancer therapy that are used clinically, however, the cardiotoxicity side effects significantly limits its use. Cardiotoxicity increases the risk of mortality and morbidity for patients under chemotherapy. Thus, treatment approaches are being advanced to avoid DOX cardiotoxicity. Many studies showed that several pharmacological agents have protecting effects against DOX cardiotoxicity [33].

Met is an oral antihyperglycemic therapy for T2DM. Met also used for weight loss, reducing plasma levels of lipid, and also prevents some vascular complications. Several studies illustrated that Met has cardioprotective effects on myocardial ischemia in experimental animal models [34].

Cardiac troponins are proteins released to the circulation when cardiac myocyte damage[23]. Troponins are the first serum biomarkers for the detection of cardiac damage. Troponins regulating the contractile elements of cardiac muscle actin and myosin. When cardiac damage occurs troponins may increase within 2-3 hours[24]. TnI levels increased in the blood stream have become a well-established biomarker with great specificity and sensitivity for myocardial infarction or necrosis in man and animals. Release of TnI from myocardium is proportional to the size and extent of myocardial injury in numerous animal models of cardiotoxicity[35].

DOX cause a significant elevation in plasma Tn I level. This elevation caused by oxidative stress that induced cardiac damage[36]. This study revealed that Met led to significant decrease ($P<0.05$) in plasma Tn I level in both Met + acute DOX and Met + chronic DOX groups.

Basnet *et al*, showed significantly reduce of cardiac TnI level by Met by activation of AMPK and endothelial NO [37]. Several studies support that Met has significant cardioprotective effects on cardiac injury in animal model studies through increasing the tolerance to ischemic damage that occurs by decreasing of AMP.

In addition, Met decreased DOX induced-cardiotoxicity through activation of AMPK[38]. AMPK regulate the cardiac metabolic pathway by catabolic and anabolic processes regulation, it reserve energy homeostasis of the heart. AMPK plays an important role in the protection of mitochondria by regulation of gene transcription and up-regulation of antioxidant endogenous system[39].

The transforming growth factor-β is a multifunctional cytokines that regulates various processes such as cell growth, tissue differentiation, apoptosis, proliferation, and migration [26]. Smads are intracellular signaling effectors which are necessary for the mediation of the TGF-β intracellular signaling[27]. Myocardial fibrosis related to increase in TGF-β/Smad signaling, as well as myocyte apoptosis [30]. So the development of cardiac fibrosis may be attributed to harmful effects of TGFβ/Smads signaling. The overexpression of inhibitory Smad7 reduced collagen synthesis, thus approving that collagen synthesis is occurred by TGFβ /Smads signaling [40].

Al-Shabanah *et al*, showed that DOX induces cardiotoxicity by increasing the expression of a specific genes in TGF-*β*/Smad pathway[41]. Janeesh *et al*, illustrated that DOX increased the expression of TGF-β1, Smad2, Smad3, Smad4 and down-regulated expression of Smad7 gene, So this showed DOX cardiotoxicity may be related to TGF-*β*/Smad signaling pathway[42].

Xiao *et al*, showed that Met inhibited cardiac fibrosis and inhibited collagen synthesis in cardiac fibroblasts by the inhibition of the TGF-β1/Smad3 signaling pathway[43].

Li *et al*, illustrated that Met inhibit TGF-β-induced fibrosis by decreasing TGF-β-induced expression of Collagen 1A1and also by decreasing the expression of Smad2 and Smad3 proteins[44].

This study showed that MET produced a significant decrease ($P<0.05$) in TGFβ1 and Smad3 level in both Met + acute DOX and Met + chronic DOX groups when compared with acute and chronic DOX groups. As a result, these data suggest that MET decreased TGF-β-induced cardiac fibrosis that appear clearly by trichrome stain result (a specific stain for collagen fiber).The TGFβ1, Smad3 and related fibrosis suppressive effect of MET may be related to the activation of AMPK. Also MET prevented TGF-β1 induced collagen synthesis independent of AMPK activation by directly binding to TGF-β1 ligand, thus blocking the binding of TGF- β1 to TβRII which lead to decrease downstream signaling [45].

CONCLUSIONS

From the results above, one can be concluded that metformin have a good cardioprotective agent against doxorubicin cardiotoxicity in both acute and chronic induction. Met showed a valuable cardioprotective effect through the suppression of serum Tn I, Smad 3 level, supressed the expression of TGF β, decreases fibrosis by activation of AMPK and increased APN level and its receptor (adipoR1 and adipoR2).

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